A new method for the preparation of an immunologically homogeneous β-casein

SUMMARY. The preparation of an immunologically homogeneous β-casein is described, involving several separations by chromatography on diethylaminoethyl cellulose with urea-imidazole buffer at pH 7, followed by Tris buffer at pH 8·2. 30 % of the β-casein present in the skim-milk is obtained by this method. β-casein gives a single band in urea-starch gel electrophoresis when it is obtained from the milk of cows homozygous for this character.

INTRODUCTION

β-casein is one of the major proteins of milk since it represents 25 % of the total milk proteins or about 30 % of the whole casein (Ribadeau-Dumas, Maubois, Mocquot & Garnier, 1963). It was one of the first caseins to be prepared in a reasonable state of purity by Warner (1944) and by Hipp, Groves, Custer & McMeekin (1952), although this latter preparation was shown by Garnier, Ribadeau-Dumas & Gautreau (1962) to contain at least 2 minor contaminants. Recently 2 other methods were proposed: Groves, McMeekin, Hipp & Gordon (1962) prepared β-casein with a very low yield (0·5–1 % of total β-casein in milk) which still presented slight impurities; Aschaffenburg (1963) proposed a method of preparation capable of giving a good yield (30 %) of β-casein, but free only of major impurities such as α_s- and κ-caseins.

A technique is described for the preparation of β-casein which also gives a good yield. The β-casein obtained is free from any contaminants which may be detected by such sensitive methods as starch-gel-urea electrophoresis or immunoelectrophoresis.

Aschaffenburg (1961) has recently observed genetic variants of β-casein. Using the technique presented here, pure genetic variants of β-casein may be prepared providing that the starting material is obtained from cows which are homozygous for β-casein. The chemical differences observed between β-casein variants will be published later.

EXPERIMENTAL

Principle

A homogeneous material is obtained, amounting to 30 % of the β-casein contained in whole casein, by chromatographic separations on DEAE cellulose in urea-imidazole buffer and then in Tris buffer.

Preparation of β-casein

10-ml fractions are collected and the purified β-casein is recovered in the salt eluate as shown in Fig. 2 while the tailing is carefully discarded. The β-casein is precipitated at pH 4·5 with 0·1 N-HCl at room temperature, washed once with distilled water, dissolved in 25-ml Tris buffer I and rechromatographed a second time in Tris buffer as above. The final β-casein is precipitated at pH 4·5 with 0·1 N-HCl, washed 3 times with distilled water, and freeze dried or alternatively dried with absolute ethanol (3 times) and ether (3 times).

Ribadeau-Dumas *et al.* (1963) found that whole casein contained about 30 % β-casein. The yield of β-casein by the method described here varies between 80 and 115 mg/g of whole casein, i.e. approximately 30 % of the theoretical yield.

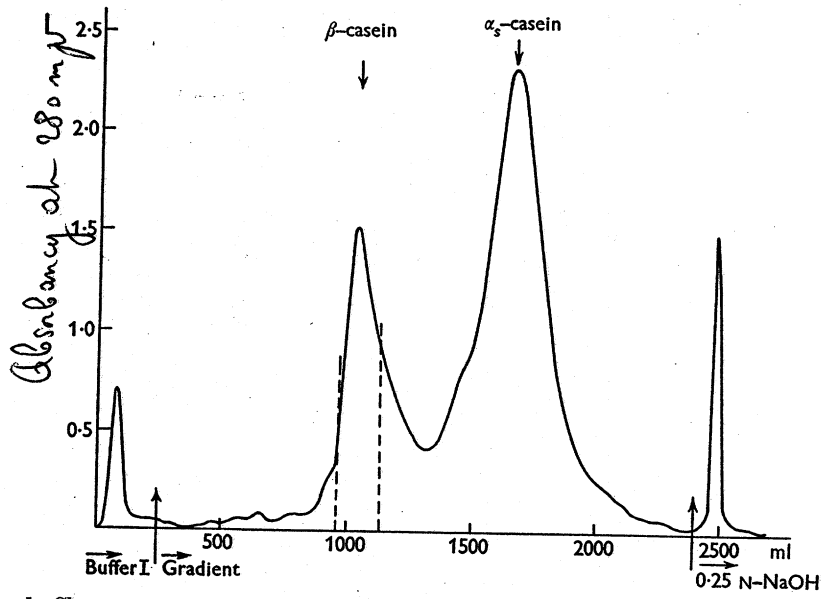


Fig. 1. Chromatography in urea-imidazole buffer at pH 7 of whole casein from the milk of 1 cow (β-caseins A and B) on DEAE cellulose.

Analytical methods

Immuno-electrophoresis was carried out according to Grabar & Williams (1955) under the conditions described by Garnier *et al.* (1962) in veronal buffer $\mu = 0{\cdot}025$, pH 8·2 and using immune rabbit serum prepared against whole casein.

Starch-gel electrophoresis was run at pH 8·6 in 7 M urea by the method of Wake & Baldwin (1961), and at pH 3·8 in 5 M urea by the method of Groves *et al.* (1962). The gels were prepared with 'Starch-Hydrolysed', obtained from the Connaught Medical Research Laboratories, Toronto, Canada.

Ultracentrifugation was performed on a mixture of β-caseins A and B in veronal buffer-NaCl ($\mu = 0{\cdot}1$) at pH 7·78 according to Sullivan *et al.* (1955) at 3 °C in a Spinco model E apparatus. The concentration of β-casein was 0·95 % (w/v).

Phosphorus determinations were made using the method of Bamann, Novotny & Rohr (1948). Nitrogen was determined by a micro-Kjeldahl method (Ogg, 1960).

Acid casein precipitation

Whole casein is prepared from the skim-milk of individual Friesian cows previously typed for their genetic β-casein variants. Casein is first precipitated from milk at pH 4·5 and then twice at pH 4·7. After each precipitation the casein is carefully washed 3 times with distilled water and dissolved at pH 7 by slowly adding N-NaOH. The solution of whole casein at pH 7 is freeze-dried.

Chromatography in urea-imidazole buffer

Essentially the same technique as the one previously described by Ribadeau-Dumas (1961) and Ribadeau-Dumas *et al.* (1963) is used except that a linear salt gradient is employed.

Whatman DEAE cellulose powder DE 50 is sieved to select particle sizes between 250 and 120 mesh. A column 3 × 16–18 cm is prepared and equilibrated with buffer I (0·01 M imidazole-4·5 M urea-pH 7). Two grams of freeze-dried whole casein are dissolved in 35–40 ml of buffer I and applied to the column. The column is eluted first with buffer I and then with a linear salt gradient up to 0·6 M-NaCl at the rate of 80 ml/h.

The linear salt gradient is obtained by putting 2 l of buffer II (0·02 M imidazole-3·3 M urea-pH 7) into the mixing vessel and 2 l of buffer II containing 0·6 M-NaCl into the other vessel.

10-ml fractions are collected and the crude β-casein, which corresponds to the first major peak, is recovered (see Fig. 1) and dialysed against 10 l of 0·05 M-NaCl solution at 4 °C for about 20 h.

Finally, the column is washed overnight at 80 ml/h with 0·25 N-NaOH, regenerated with 0·1 M piperazine-HCl (pH 4·4)–20 % NaCl for 24 h and re-equilibrated with buffer I till the pH rises to 7. This step requires about 48 h.

The 4·5 and 3·3 M urea solutions are treated before use with activated charcoal to remove material absorbing at 280 mμ. Activated charcoal (1 g/l) is added to the urea solutions, and after stirring for 30 min the solutions are filtered, first on paper, then on a 3 × 10 cm column of DEAE cellulose. They are then made up to 0·01 or 0·02 M imidazole, and the pH is adjusted to 7 with concentrated HCl.

Chromatography in Tris buffer

After dialysis the crude β-casein is precipitated at pH 4·7 with 0·1 N-HCl at room temperature, washed once with distilled water, dissolved in 25 ml of Tris buffer I (0·01 M tris (hydroxymethyl) aminomethane adjusted to pH 8·2 with concentrated HCl). During dissolution the pH is maintained at pH 8·2 with 0·25 N-NaOH. The solution is then applied to a 3 × 15 cm Whatman DEAE cellulose column, similar to the one described above, previously equilibrated with Tris buffer I. The column is eluted first with Tris buffer I then with an exponential salt gradient at the standard rate of 80 ml/h.

The exponential salt gradient is obtained by adding a solution of Tris buffer II (0·02 M Tris, pH 8·2) with 0·6 M-NaCl dropwise to the mixing chamber containing 2 l of Tris buffer II. Finally the column is washed with 0·25 N-NaOH and then with 0·1 M piperazine-HCl (pH 4·4)–20 % NaCl, as for chromatography in urea, and re-equilibrated with Tris buffer I.

Preparation of β-casein

Usually a second chromatography in the same conditions is needed to obtain an immunologically homogeneous β-casein (Fig. 3*c*). It is unlikely that the 'tail' prolonging the main arc of precipitation is due to an impurity since there is a complete immunological cross-reaction with β-casein and only a single band is observed on starch gel electrophoresis (Plate 1*c*).

The homogeneity of the β-casein was also checked by the double diffusion technique (Kaminsky, 1954), with various β-casein concentrations from 0·06 to 2 %.

The purity of the β-casein obtained depends on the quality of the DEAE cellulose used. For instance, with some commercially available samples of DEAE cellulose we failed to get pure β-casein, although using the chromatographic schedule described above. A good test for the suitability of this ion exchanger is its ability to demonstrate the presence of minor constituents appearing between the β-casein peak and the α_s-casein peak during chromatography of whole casein in urea (Fig. 1). All attempts to increase the yield of homogeneous material above 30 % have failed. To ensure the purity of the final preparation it is necessary to collect only those fractions which strictly correspond to the β-casein peak (see dotted lines in Figs. 1 and 2).

There is a small but definite difference in mobility at pH 8·2 in veronal buffer between the A, B and C genetic variants of β-casein. However, the resolving power of chromatography in Tris buffer is still not sufficient to separate the genetic variants although the asymmetry of the peak suggests a slight degree of separation. Consequently, the genetic variants can at present only be obtained by chromatography of the caseins of individual samples of milk from cows homozygous for β-casein.

Starch gel electrophoresis diagrams of samples of β-caseins obtained from individual samples of milk at 2 pH values and at various concentrations are presented in Plate 1. One of the preparations (Plate 1*a*, *b*) comes from a cow which was heterozygous for β-casein, and gives 2 bands corresponding to β-casein A and β-casein B. The other preparation (Plate 1*c*) comes from a cow which was homozygous, and gives a single band. Both preparations are free from contaminants even at a concentration as high as 2 %.

Preliminary experiments performed on β-casein A, B and C showed a complete cross reaction between them by the techniques of double diffusion in agar and immunolectrophoresis. The same result was found by Gough & Jenness (1962) for β-lactoglobulins A and B.

A small amount of sialic acid was found in β-casein A or AB, i.e. 0·018–0·03 %. This could be explained by a trace of κ-casein tightly bound to β-casein, and not detectable by our techniques, or by a possible interference of the protein itself in the determination of sialic acid since an excess of β-casein had to be used to allow the small amount of sialic acid to be measured. Such a small amount has also been found in purified α_s-casein by Schmidt & Payens (1963).

The ultracentrifugation diagram of the purified β-casein showed a single symmetrical peak which according to Payens & van Markwijk (1963) corresponds to the monomer of β-casein. The sedimentation coefficient $s_{20,w}$ obtained was $1{\cdot}22 \times 10^{-13}$ sec.

The phosphorus content of 0·52 % for β-casein A agrees with 4 atoms of phosphorus for a molecular weight of 25,000 determined by Payens & van Markwijk (1963) and the nitrogen content was 14·4 %.

Sialic acid was determined by the thiobarbituric acid method of Warren (1959). The reference sample was prepared from whole casein according to Ribadeau-Dumas & Alais (1961).

Dry weight determinations were made by drying under vacuum with P_2O_5 at room temperature for 24 h.

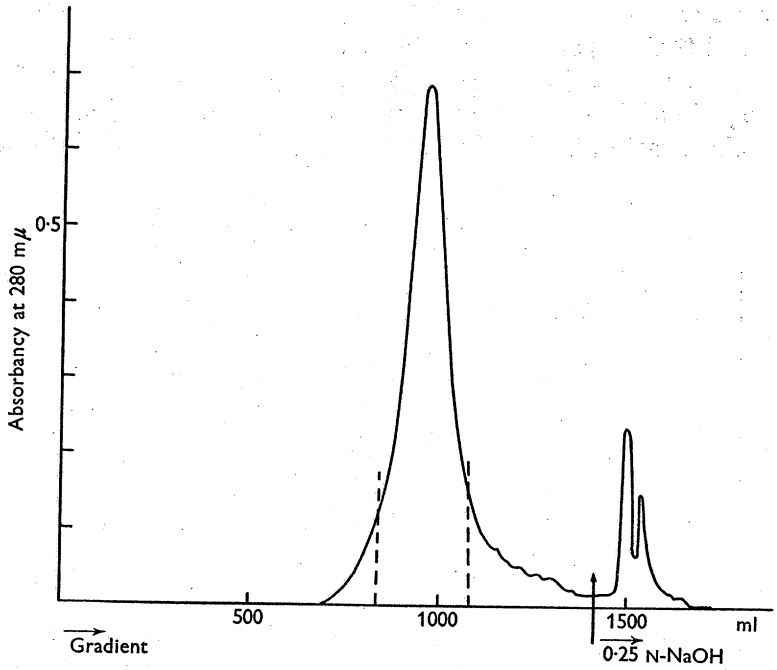


Fig. 2. First chromatography of crude β-casein in Tris buffer at pH 8·2 on DEAE cellulose.

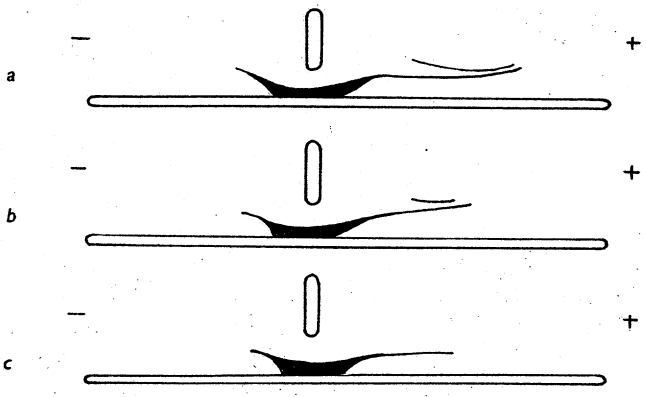
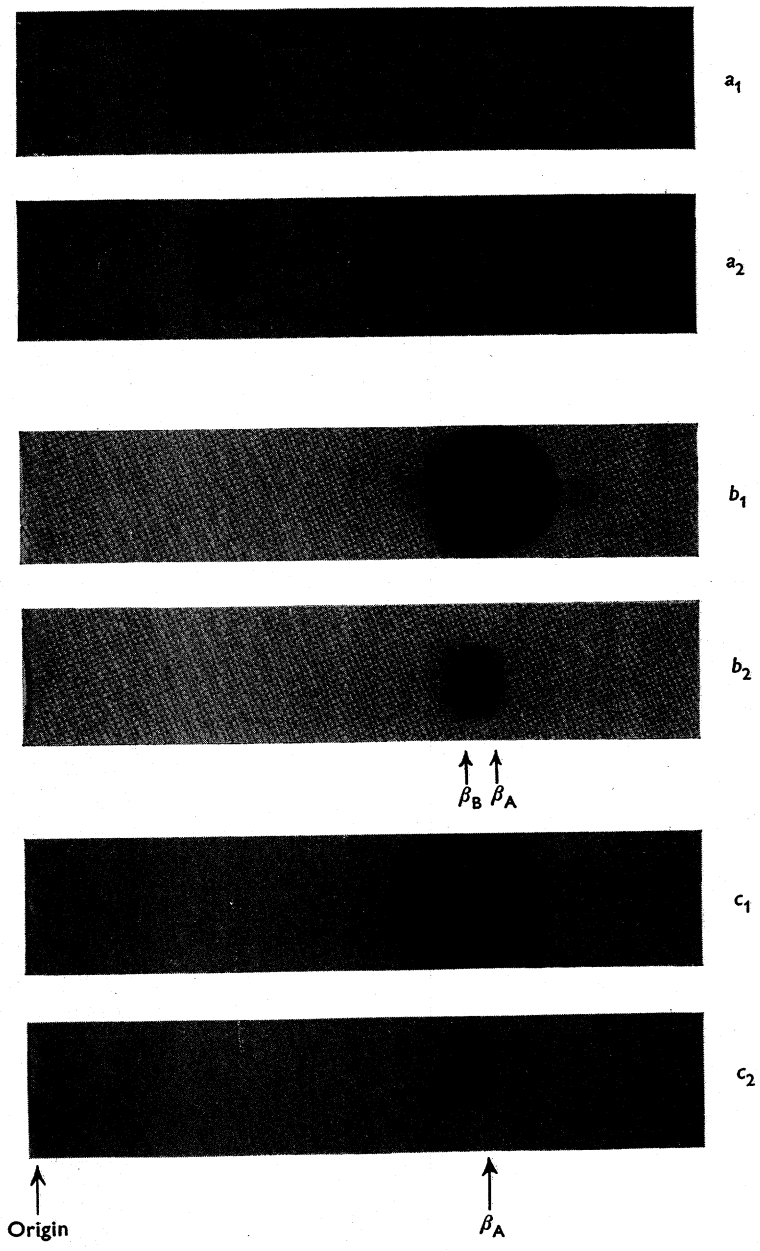


Fig. 3. Immunoelectrophoretic diagrams of: (*a*) crude β-casein after urea-imidazole chromatography; (*b*) β-casein after the first chromatography in Tris buffer at pH 8·2; (*c*) final β-casein preparation after rechromatography in Tris buffer at pH 8·2. Concentration of β-caseins, 0·5 % (w/v).

RESULTS AND DISCUSSION

Chromatography in Tris buffer (pH 8·2) was carried out to remove the fast moving immunoelectrophoretic component which contaminates crude β-casein preparations (Fig. 3*a*). This impurity is an α_s-casein fraction other than α_s-1, 2.


a_1
a_2
b_1
b_2
β_B β_A
c_1
c_2
Origin
β_A

The extinction coefficient at 278 mμ in phosphate buffer, pH 7 ($\mu = 0{\cdot}1$, for a 1 % solution in a 1 cm path cell) has been found to be $E\,\frac{1\%}{1\,\mathrm{cm}} = 4{\cdot}6$ for β-casein A.

Chromatographic separations can be easily mechanized and in the near future automatic protein analysers will be available. The preparation of large amounts of purified casein fractions by chromatography will then become possible due to the high resolving power of this technique.

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EXPLANATION OF PLATE

PLATE 1

Starch gel electrophoresis of β-casein in urea. (*a*) β-caseins AB, pH 3·8, in 5 M urea at concentrations of 2 % (a_1) and 0·2 % (a_2); (*b*) β-caseins AB, pH 8·6, in 7 M urea at concentrations of 2 % (b_1) and 0·1 % (b_2); (*c*) β-casein A, pH 8·6 in 7 M urea at concentrations of 2 % (c_1) and 0·1 % (c_2).